EFFECT OF CAFFEINE ON DNA SYNTHESIS IN MAMMALIAN CELLS

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ABSTRACT Alkaline sucrose sedimentation studies of DNA from mouse lymphoma cells (L5178Y) treated with caffeine have demonstrated the following effects. Caffeine (at a concentration of 1.6 mM) does not introduce strand breaks into preformed DNA nor does it inhibit the rejoining of γ-ray-induced strand breaks. Although it does not affect the over-all rate of DNA synthesis, pulse labeling experiments show that the DNA strands synthesized in its presence are smaller than those made in its absence. This could be the result of (*a*) DNA being made in shorter replicating units or (*b*) small gaps in the daughter DNA strands within normal-sized replicating units. These two alternative models were indirectly distinguished as follows. After a pulse label with thymidine-^{3}H in the presence of caffeine, cells were incubated without caffeine in bromodeoxyuridine (BrdUrd). During this incubation, growing strands are elongated and hypothetical gaps (model *b*) filled in with bromuracil (BrUra)-substituted DNA. The BrUra-containing DNA segments will now be of different lengths on the two models. With smaller replicating units (*a*) the "elongation segments" will be somewhat smaller than but the same order of magnitude as those in untreated cells, whereas with small gaps (*b*) the "filled-in gap segments" would be expected to be at least an order of magnitude smaller. The BrUra-containing regions of DNA can be selectively broken open by exposing the cells to light at 313 nm. The exposure required to break open the BUra-substituted regions is inversely related to, and hence gives a measure of, the size of these regions. In caffeine-treated cells these regions were found to be somewhat smaller than but of comparable size with those in untreated cells; this is consistent with the DNA being synthesized in smaller units and argues against the presence of small gaps in the daughter strands.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) has been frequently used in studies of mutation, toxicity, and radiosensitization of mammalian cells. At concentrations in the region of 10^{-3} M the drug is not toxic to L cells but sensitizes them to ultraviolet (UV) irradiation (Rauth, 1967; Domon and Rauth, 1969) and various alkylating agents (Rauth et al., 1970; Walker and Reid, 1971). This effect is not, however,

seen with HeLa cells (Wilkinson, et al., 1970) and reports with Chinese hamster cells are conflicting (Arlett, 1967; Cleaver, 1969 *a*; Trosko and Chu, 1971). The sensitizing effect on L cells appears to be exerted during the DNA synthesis period immediately after the radiation (Rauth, 1967; Domon and Rauth, 1969).

At concentrations in excess of 1–2 $\times$ 10^{-3} M, caffeine is toxic to L cells (Rauth, 1967), Chinese hamster cells (Arlett, 1967; Cleaver, 1969 *a*; Trosko and Chu, 1971), and HeLa cells (Kuhlmann et al., 1968), and induces chromosome aberrations in HeLa cells and leukocytes (Kuhlmann et al., 1968).

The experiments described below were begun in order to investigate the manner in which caffeine exerts its radio-sensitizing action, at the level of DNA replication, since an earlier report by Cleaver and Thomas (1969) had suggested that it might specifically inhibit the joining up of gaps present in daughter DNA strands made in UV-irradiated cells. It was found, however, that in mouse lymphoma L5178Y cells, caffeine at a concentration of 1.6 mM affected DNA synthesis even in unirradiated cells and it was considered important to investigate this effect before turning to studies with UV-irradiated cells. This paper is an attempt to formulate the effects of caffeine on DNA synthesis and to assess the significance of these effects with regard to UV sensitization by caffeine. The results show that although caffeine has no effect on the size of preformed DNA nor on the over-all rate of DNA synthesis, the DNA strands synthesized in its presence are smaller than those made in its absence. One possible explanation for this observation for which some evidence will be presented is that, in the presence of caffeine, DNA is synthesized in smaller replicating units than in untreated cells. A model which accounts for most of the reported effects of caffeine will be presented.

MATERIALS AND METHODS

Mouse lymphoma L5178Y cells were used in these experiments as in previous studies (see Lehmann and Ormerod, 1970; Lehmann, 1972, for details of growth conditions). They were kindly supplied by Dr. G. A. Fischer. DNA inside the cells was radioactively labeled by incubating cells with thymidine-2-^{14}C (50 mCi/mmole) or thymidine-methyl-^{3}H (15–20 Ci/mmole). Caffeine (Calbiochem, Los Angeles, Calif.) when present was usually at a concentration of 0.3 mg/ml (1.6 mM). Techniques for pulse labeling with thymidine, chasing with BrdUrd, and the exposing of cells to light at 313 nm have been described elsewhere (Lehmann, 1972). In pulse labeling experiments in the presence of caffeine, cells were always preincubated with caffeine for 30 min before adding the thymidine-^{3}H.

Alkaline sucrose gradient sedimentation was carried out as follows. After incubation, cells were resuspended in a balanced salt solution and exposed to 2 krads of γ-irradiation (from a ^{60}Co source) to prevent entanglement of DNA strands on subsequent denaturation (Lehmann, 1972). 1–2 $\times$ 10^{4} cells in 25–50 μl were immediately lysed in 2% sodium dodecyl sulphate/0.02 M ethylenediaminetetraacetate (EDTA) on top of a 4 ml 5–20% alkaline sucrose gradient containing 0.1 N NaOH and 0.1 M NaCl. The gradients were spun in an SW 56 rotor in a model L centrifuge (Beckman Instrument, Inc., Spinco Div., Palo Alto, Calif.) at 40,000 rpm for 75 min. Fractions were collected on paper strips (Carrier and Setlow, 1971), washed, and cut up, and the radioactivity was counted as described previ-

ously (Lehmann, 1972). Molecular weights when required were calculated by computer program (Regan et al., 1971; Lehmann, 1972).

RESULTS

Rate of DNA Synthesis

Fig. 1 shows that the presence of 0.3 mg/ml caffeine does not alter the rate of DNA synthesis in L5178Y cells, as judged by incorporation of thymidine-^{3}H into the acid-insoluble fraction of the cells. A similar finding has been reported for HeLa cells (Kuhlmann et al., 1968). Prelabeling of the cells with thymidine-^{14}C enabled sampling errors to be eliminated, so that small differences in rates of uptake could have been detected.

Effects of Caffeine on Preformed DNA

In these experiments cells were labeled for 16 hr with 0.5 μCi/ml thymidine-^{14}C and then incubated for 1 hr in the presence or absence of 0.3 mg/ml caffeine. No difference was observed in the radioactivity sedimentation profiles on alkaline sucrose gradients between the DNA from treated and untreated cells (results not shown). If after incubation in caffeine, the cells were exposed to 20 krads of γ-irradiation and then incubated for a further 30 min at 37°C in the presence of the drug, the rate of rejoining of the γ-ray induced single strand breaks was the same as that in cells which had not been exposed to caffeine (Fig. 2). It may be concluded from

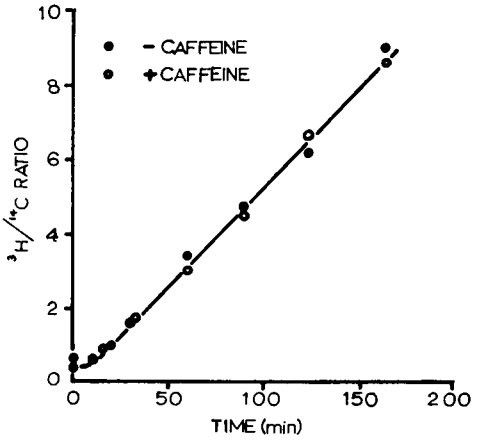


FIGURE 1 Effect of caffeine on rate of DNA synthesis. L5178Y cells were grown for 16 hr in the presence of 0.5 μCi/ml thymidine-^{14}C. After washing out the thymidine, the cells were incubated with or without 0.3 mg/ml caffeine. After 30 min 25 μCi/ml thymidine-^{3}H was added to each tube and 25 μl samples removed at various times onto paper disks soaked in 2% sodium dodecyl sulphate. The ^{3}H and ^{14}C counts in the trichloroacetic acid-insoluble fractions of the cell were measured. The ratio of ^{3}H/^{14}C counts gives a precise measure of the average amount of synthesis per cell and eliminates sampling errors.

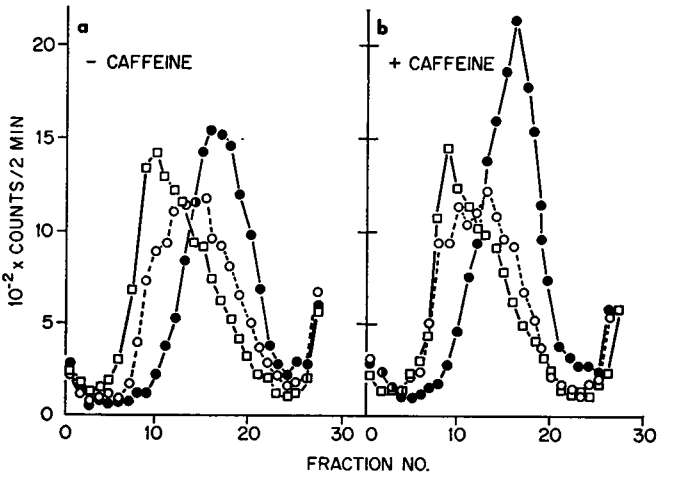


FIGURE 2 Effect of caffeine on rejoining of γ-ray induced strand breaks. L5178Y cells were grown for 16 hr in the presence of 0.3 μCi/ml thymidine-^{3}H. After washing to remove the radioactive label the cells were incubated for 30 min in the presence or absence of caffeine (0.3 mg/ml), then resuspended in saline and exposed at 0°C to 20 krads of γ-irradiation (dose rate: 4 krads/min). After irradiation the cells were diluted into prewarmed medium and incubated further in the presence or absence of 0.3 mg/ml of caffeine. Samples were taken 0 (●——●), 10 (○---○), or 30 (□——□) min after irradiation, resuspended in saline at a concentration of 5–10 × 10^5 cells/ml and 25 μl layered into 0.1 ml detergent on top of 3.9 ml alkaline sucrose gradients. The gradients were subsequently centrifuged at 40,000 rpm for 75 min, fractions collected, and their radioactivity counted. Sedimentation is from right to left.

these results that caffeine neither produces strand breaks in DNA, nor does it inhibit their rejoining.

Size of Newly Made DNA

The size distributions of DNA strands synthesized in the presence and absence of caffeine were compared by analyzing the alkaline sucrose gradient profiles of DNA from cells pulse labeled for 30 min in the presence or absence of the drug so that only growing strands became labeled (Fig. 3). In this case there is a marked difference, the profiles from the caffeine-treated cells being shifted towards the top of the gradient (Fig. 3 *a*). Thus the DNA strands which are being synthesized in the presence of caffeine are smaller than those made in its absence, the effect being dose dependent (Figs. 3 *a* and 3 *b*). The variation between experiments (Fig. 3 *b*) is probably caused by differences in the state of the cells, effective pulse time, washing procedures, etc. Within one experiment, however, there was always a decrease in molecular weight with increasing concentrations of caffeine.

In untreated cells a cold chase after a half-hour pulse label causes a shift of the profiles to higher molecular weights corresponding to elongation of growing strands and joining up of adjacent replicating units (Lehmann, 1972). As shown in Fig. 3 *c*

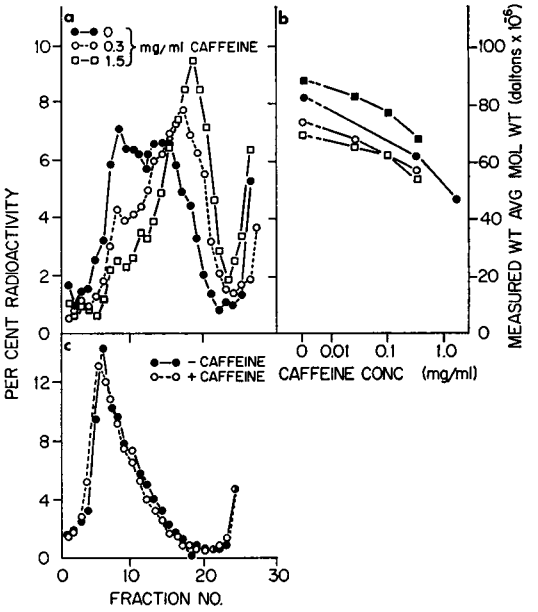
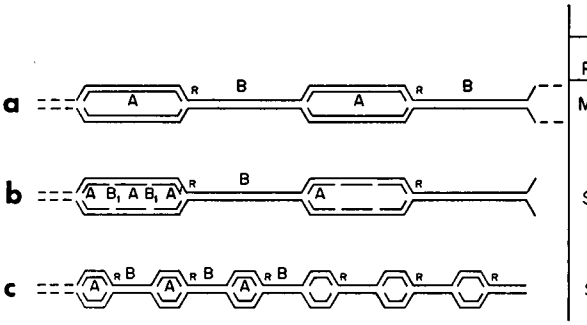


FIGURE 3 Effect of caffeine on size of newly synthesized DNA. (*a*) Cells were incubated for 30 min with the indicated concentrations of caffeine. They were then pulse labeled for 30 min (the drug still being present) with 50 μCi/ml thymidine-^{3}H, and alkaline sucrose gradient centrifugation carried out as described in Materials and Methods and in the legend to Fig. 2. (*b*) Plot of measured weight average molecular weights calculated from sedimentation profiles of DNA from cells pulse labeled for 30 min with thymidine-^{3}H in different concentrations of caffeine. (Since the DNA is not uniformly labeled, these values do not represent true weight averages.) Different symbols represent different experiments. (*c*) Sedimentation profiles of DNA from cells preincubated for 30 min in the presence or absence of 0.3 mg/ml caffeine, pulse labeled for 30 min with 50 μCi/ml thymidine-^{3}H in the presence or absence of caffeine and chased for 2 hr with 10^{-5} M thymidine and 10^{-5} M deoxycytidine in the *absence* of caffeine. Sedimentation from right to left.

a 2 hr cold chase in the absence of caffeine after a half-hour pulse label in the presence or absence of caffeine produced identical profiles, showing that the effect of caffeine is reversible on removing the drug.

Nature of Newly Made DNA

The low molecular weight shift in the profiles of DNA synthesized in the presence of caffeine could be the result of (*a*) gaps left in the newly synthesized strands of DNA, similar to those seen opposite pyrimidine dimers in newly synthesized strands in UV-irradiated cells (Rupp and Howard-Flanders, 1968; Zipser and Rupp, 1970; Lehmann, 1972), or (*b*) the synthesis of DNA in smaller replicating units. Gaps in the daughter strands would result in DNA containing small single strand regions. Attempts to test for this by Cs_2SO_4/Hg (II) equilibrium density centrifugation, or with benzoylated naphthoylated *o*-(diethylaminoethyl)cellulose chroma-



	PROFILE TYPES			
			313 nm EXPOSURE	
	PULSE	CHASE	LOW	HIGH
a	MEDIUM	LARGE	MEDIUM	MEDIUM
b	SMALL	LARGE	MEDIUM	SMALL
c	SMALL	LARGE	SMALL	SMALL

FIGURE 4 Models for mode of action of caffeine and expected sedimentation profiles. The left part of the diagram represents models for replicating DNA from untreated cells (*a*) or cells growing in the presence of caffeine (*b*) and (*c*). In (*b*) small gaps, B_1 are left in the growing strands, whereas in (*c*) DNA is synthesized in shorter replicating units than in untreated cells. In a pulse labeling experiment growing strands *A* become labeled at their ends at the replication points *R*. The right-hand part of the figures shows expected profile types, termed large, medium, or small (see text), after a 30 min pulse label with thymidine-^{3}H (pulse) followed by a 2 hr cold chase with BrdUrd (Chase) and exposure to low or high doses of radiation at 313 nm.

tography, however, were unsuccessful, since I found, in confirmation of other work (Paoletti et al., 1967; Levis et al, 1967; Kidson, 1968) that newly synthesized DNA, even in untreated cells, appeared to have a partially denatured character. Hence a less direct method of distinguishing between alternatives (*a*) and (*b*) was used. The rationale behind this method is shown in Fig. 4 and discussed below. Replicating DNA in mammalian cells is thought to have the structure shown in Fig. 4 *a* (Huberman and Riggs, 1968; Lehmann and Ormerod, 1972). Replicating units are joined end-to-end and in L5178Y cells are approximately 2×10^8 daltons in length. In a short pulse labeling experiment the new strands *A* (Fig. 4) become labeled at their ends at the replication points *R*. Since in an asynchronous population cells will be at all stages of the cell cycle and in particular at all stages of the S period, the average spacing (*B* in Fig. 4) between growing DNA strands will be about half a replicating unit. If in the presence of caffeine the DNA contained small gaps there would be (see Fig. 4 *b*), in addition to spacings *B*, much smaller spacings B_1 (the gaps) within the new strands. With smaller replicating units however (Fig. 4 *c*), the average spacings *B* should be smaller than but of the same order of magnitude as those in untreated cells.

The size of the spacings in DNA inside cells may be estimated by taking advantage of the selective photolysis of BrUra-containing DNA by light at 313 nm (Regan et al., 1971). If the spacings are filled in with BrUra-substituted DNA by incubating the cells in BrdUrd they can be subsequently reopened by exposure of the cells to light at 313 nm, which selectively breaks BrUra-substituted segments of DNA. Furthermore, to a first approximation, the dose of 313 nm light necessary to introduce a break into BrUra-substituted segment is inversely proportional to

the size of the segment. Hence by determining the dose of 313 nm light needed to disrupt such a stretch, its length can be estimated. This technique has been previously used by Regan et al. (1971) to measure the size of gaps filled in by repair replication after the excision of pyrimidine dimers in UV-irradiated cells, and by Lehmann (1972) to measure gaps in daughter strands opposite unexcised pyrimidine dimers, and is explained in greater detail in these papers.

Cells were therefore pulse labeled with thymidine-^{3}H in the presence or absence of caffeine, then chased for 2 hr with medium lacking caffeine and containing BrdUrd. Subsequently they were exposed to light at 313 nm and the DNA centrifuged in alkaline sucrose.

The expected radioactivity profiles at various stages of the procedure for untreated cells and treated cells on the two models are shown at the right of Fig. 4. The pulse label radioactively labels growing strands *A* at their ends and produces profiles like those shown in Fig. 3 *a*, termed "medium" for untreated cells and "small" for treated cells. Incubation in BrdUrd would result in all the spacings *B* and B_1 being filled in with BrUra-substituted DNA, giving in all cases profiles like those shown in Fig. 3 *c*, and termed "large." On exposure at 313 nm relatively low doses of light will disrupt the relatively large filled-in elongation segments, labeled *B*, but much higher doses will be needed to reopen the much smaller filled-in gaps B_1, so that in the gap model a biphasic dose response is expected. Hence the two models differ in the doses of 313 nm light needed to reform the original profiles (i.e., small for treated and medium for untreated cells). These doses are of the same order of magnitude for treated and untreated cells on the "small units" model, though some difference is expected. Much higher doses are needed for the treated than for the untreated cells on the "small gap" model. In the experiments it was found (see Fig. 5) that the production of small profiles from treated cells occurred at exposures of 313 nm light only slightly greater than those necessary to reform medium profiles from untreated cells. By reference to Fig. 4 it can be seen that these results are consistent with the "smaller replicating units" model (Fig. 4 *c*) and incompatible with a DNA structure containing small gaps (Fig. 4 *b*). Such small gaps are seen in DNA synthesized in UV-irradiated cells and in comparable experiments they are only reopened by exposures at 313 nm of more than 10^5 ergs/mm^2 (Lehmann, 1972).

The data of Fig. 5 show an appreciable amount of experimental scatter. Since the above conclusions are based on relatively small molecular weight differences, it is perhaps necessary to comment on this experimental scatter. The results shown in Fig. 5 are taken from four separate experiments and as shown below, the scatter is almost entirely due to variations in molecular weights between experiments rather than within one experiment. Let the ratios of the molecular weights in the +caffeine and the −caffeine samples after a given dose of 313 nm light (the "final caffeine effect") be *p*, and the ratio after the pulse label with no further treatment (the "initial caffeine effect") be *q*. In six separate experiments the mean value of

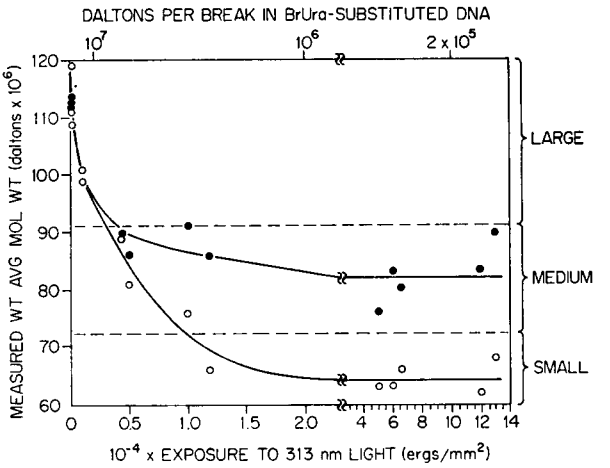


FIGURE 5 Breakdown of DNA by light at 313 nm. Cells were pulse labeled in the presence (○) or absence (●) of 0.3 mg/ml caffeine, followed by a 2 hr cold chase with 5 μg/ml BrdUrd, 2.5 μg/ml deoxycytidine. The cells were resuspended in balanced salt solutions and irradiated in a quartz cuvette with light at 313 nm from a monochromator illuminated by a mercury arc light. Molecular weights were calculated for the DNA from the irradiated cells by performing alkaline sucrose sedimentation experiments. Note the change in scale of the abscissa. The upper abscissa is a conversion of the 313 nm exposure into daltons per break in BrUra-substituted DNA and is obtained from a previous calibration experiment (Lehmann, 1972).

q, $\bar{q}$ = 0.81 ±0.06; the molecular weights obtained after nine high 313 nm exposures (greater than 10^4 ergs/mm²) taken from these same six experiments gave $\bar{p}$ = 0.80 ±0.05. If for these nine dose points we calculated p/q using the values of p and q from the same experiment, we obtain $(\overline{p/q})$ = 0.98 ±0.03. We may draw three conclusions: (*a*) $\bar{p}$ is significantly less than 1; this implies that small profiles do indeed have a lower molecular weight than medium profiles. (*b*) $(\overline{p/q})$ is not significantly different from 1; this shows that exposures at 313 nm of greater than 10^4 ergs/mm² produce a final caffeine effect of the same size as the initial caffeine effect. (*c*) The root mean square error of $(\overline{p/q})$ is less than that of $\bar{p}$ or $\bar{q}$; this shows that variations in the magnitude of the final caffeine effect between experiments are the result of corresponding variations in the initial caffeine effect. Within one experiment there is much less scatter.

DISCUSSION

The results presented above show that, although caffeine (at a concentration of 1.6 mM) neither introduces breaks into DNA nor inhibits the rejoining of broken DNA chains, DNA strands synthesized in the presence of caffeine are smaller than those made in its absence. In UV-irradiated cells in which newly synthesized strands show a similar displacement to low molecular weight, this shift appears to be the result

of gaps of a few hundred nucleotides left opposite pyrimidine dimers (Lehmann, 1972). In caffeine-treated cells, however, the explanation appears to be different; circumstantial evidence has been produced to show that caffeine causes the DNA to be synthesized in short replicating units. Firstly, unlike in UV-irradiated cells, DNA synthesis in the presence of caffeine takes place at the normal rate. Also the size of the spacing between adjacent strands of DNA appears to be some two orders of magnitude higher than that in UV-irradiated cells. A gap (i.e., single strand region) of this size is most unlikely and the results have therefore been interpreted as indicating the existence of some kind of abnormal synthesis involving shorter replicating units. This model is probably not the only one which can account for the experimental findings but it is consistent with the results presented above. In any case it is important that the shift to low molecular weight of DNA synthesized in the presence of caffeine is taken into account when studying its molecular mode of action in UV-irradiated cells.

MODEL FOR GENERAL ACTION OF CAFFEINE

The results of Rauth and coworkers (Rauth, 1967; Domon and Rauth, 1969) showing that caffeine only exerts its radiosensitizing effect during DNA synthesis after irradiation, and of Kuhlmann et al. (1968) that the production of chromosome breaks is only effected if caffeine is present during DNA synthesis, are consistent with my findings that caffeine only affects DNA synthesized in its presence but has no effect on preformed DNA. A speculative model, which is by no means proven, will now be put forward to account for these and other experiments with caffeine. This model is a modification of that postulated by Domon et al. (1970). It is known that caffeine binds to denatured DNA but not to native DNA (T'so et al., 1962; Domon et al., 1970). It is envisaged that this binding is reversible and dependent on the concentration of caffeine and the size of the denatured regions. The DNA replication point is in a region of partial denaturation (see Results and Paoletti et al., 1967; Levis et al., 1967; Kidson, 1968) so that there is a probability that caffeine may bind here, causing premature termination of synthesis and recommencement elsewhere. This would result in DNA being synthesized in smaller units and the effect would be concentration dependent (as shown in Fig. 3 *b*). At high concentrations this aberrant synthesis may be harmful and lead to chromosome aberrations (Kuhlmann et al., 1968) and cell death. (It is of interest to note here that Wragg et al. [1967] showed that caffeine inhibited DNA polymerase activity in mammalian cell extracts.)

In UV-irradiated cells, during DNA synthesis, gaps of about 800 nucleotides are left opposite unexcised pyrimidine dimers (Lehmann, 1972), so that caffeine would have a relatively high probability of binding here. This could cause a slowing down of the gap-filling process and some evidence for this has been put forward by Cleaver and Thomas (1969). This could then account for the radiosentitizing effect of caffeine

on rodent cells as suggested by Domon et al. (1970). Human cells unlike rodent cells are capable of efficiently excising pyrimidine dimers (Regan et al., 1968), so that the post-replication gap-filling repair process is presumably of less importance in human cells. This may explain why caffeine does not sensitize HeLa cells to UV light (Wilkinson et al., 1970).

The production of single strand breaks, either by γ-irradiation, or by incision near a pyridimidine dimer after UV-irradiation in excision-proficient cells, might produce single-stranded regions but these would probably be very limited in length, a few nucleotides at most. Hence processes of strand break rejoining (see Results), excision of pyrimidine dimers (Regan et al., 1968), and repair replication (Cleaver, 1969 *b*) are insensitive to caffeine.

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